REEF MANAGEMENT AND SEAFOOD MONITORING PROGRAMS FOR CIGUATERA

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Ciguatera (CTX) toxins in fishery products are odorless, tasteless, and generally undetectable by simple chemical test; bioassays traditionally monitor suspect fish. Assurance that susceptible foods are safe to eat will come from marketplace screening, separation of adulterated product to less risk uses, and, where feasible, prediction of potentially hazardous food production/harvesting areas. An effective screening method for use in the marketplace must be: (a) easy to use and interpret; (b) able to test a large number of samples in a short period; (c) accurately differentiate between toxic and non-toxic product; (d) low cost; (e) available in sufficient quantity to meet private, industrial, and regulatory agency demands; and (f) where possible identify toxins involved. The solid-phase immunobead assay (S-PIA, Ciguatect™) has the highest potential for this purpose. The kit can be used on fishing vessels, at receiving docks, processing plants, distribution organizations, retail outlets, consumers, and regulatory agencies and is designed for non-laboratory use by untrained personnel.

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Ciguatera fish poisoning is a centuries old illness, endemic to tropical and subtropical areas (WHO,1984), sometimes shipped to nontropical population centres (North America). Humans are exposed through consumption of fish which have accumulated toxins produced by dinoflagellates. An estimated 50,000–500,000 cases of ciguatera occur each year (Ragelis, 1984). Symptoms are gastrointestinal, neurological and cardiovascular and can persist for weeks and even years (Juranovic & Park,1991). U.S. public health agencies (Food and Drug Administration and National Marine Fisheries Service) have been striving to implement a combative seafood safety program for years.

Public health research on ciguatera has focused on protection of human health and enhancement of commerce in subtropical reef fish. To set up an effective seafood monitoring program, it is necessary to understand how products become toxic and so develop an analytical technique for detection. Historically, methods of analysis for ciguatoxins (CTX) have been labor-intensive, time-consuming, and not able to identify individual toxins (Juranovic & Park,1991).

TOXIN PRODUCING ALGAE AND PRINCIPAL TOXINS INVOLVED IN CIGUATERA

CTX accumulates in benthic feeding herbivorous fish and then up the food chain to man. Benthic toxigenic dinoflagellates suspected in ciguatera poisoning include *Gambierdiscus toxicus*, *Prorocentrum lima*, *P. concavum*, *P. emarginatum*, *P. mexicanum*, *P. rathynum*, *Amphidinium carterae*, *Ostreopis ovata*, *O. siamensis*, *O. lenticula*, *Coolia monotis*, *Scrippsiella subsalsa*, and *Thecadinium* sp.

Of several toxins which may be responsible for ciguatera, ciguatoxin has been isolated as the major toxin from large carnivores while smaller amounts have been detected in herbivores. An explanation for this could be that CTX accumulates preferentially in large carnivores due to its greater lipid solubility. Murata et al. (1990) reported the structures of ciguatoxin from the moray eel (*Gymnothorax javanicus*) and its likely precursor from *G. toxicus*. The congener was shown to be a less oxygenated analog of ciguatoxin. However, it has not been demonstrated that the toxin produced by the dinoflagellate is the precursor to ciguatoxin(s) accumulating in fish. Until sufficient quantities of individual toxins become available and suitable detection methods for these toxins are developed, it will be difficult to determine toxin properties. At least five toxins are implicated in ciguatera; they are ciguatoxin (CTX), maitotoxin (MTX), scaritoxin (STX), okadaic acid (OA), and a recently named toxin, prorocentrolid (Bagnis et al.,1974; Chungue et al.,1977; Maulin et al., 1992; Tachibana, 1980; Tindall et al.,1984; Yasumoto et al.,1971; Yasumoto et al.,1984; Yasumoto & Murata,1988a; Yasumoto & Murata,1988b; Yasumoto & Scheuer,1969). Recent studies suggest that in ex-

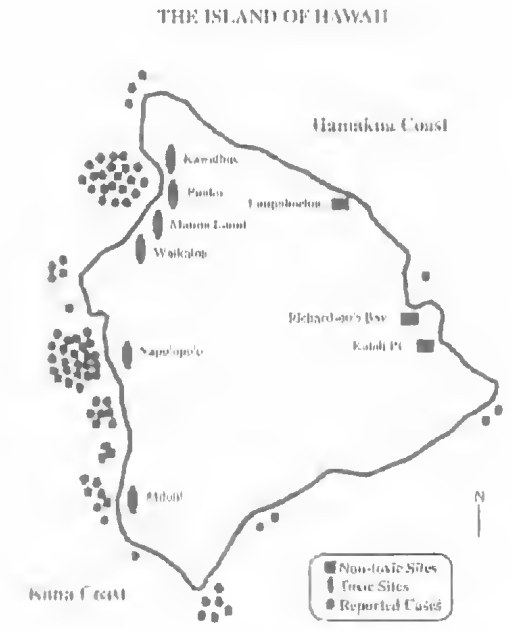

FIG. 1. Reports of cases of ciguatera fish poisoning outbreaks recorded by Hawaii State Department of Health (1981-1990). Toxic (Kona Coast) and non-toxic (Hamakua Coast) sites for collecting biomarker model are noted.

cess of 20 toxins may be involved in the ciguatera phenomenon (Juranovic et al.,in press; Legrand,1991; Lewis et al.,1991; Lewis & Sellin,1992; Lewis,1992). Relative concentrations and toxin profiles for each toxic fish vary greatly and are unknown due to the lack of individual toxin reference standards and specific analytical methods.

Okadaic acid is available commercially as a standard reference material. For CTX, however, ciguatoxin and possibly maitotoxin and their analogs are the toxins with the highest toxic potentials. The toxic potential of okadaic acid is several orders of magnitude lower than ciguatoxin.

ANALYTICAL METHODOLOGY

Analytical methods for phycotoxins vary according to the application, i.e., screening, identifying, etc. (Park,1994). For ciguatera biossays have been used in the laboratory but are unsuitable as a marketplace test. Most earlier methods were based on biological endpoints which had major limitations on levels of detection and specificity. Many native tests for fish toxicity have been examined, including discoloration of silver coins, or copper wire, the repulsion of flies or ants, and rubbing the liver on the gums to ascertain if it causes a tingling feeling (Juranovic & Park,1991). With the possible exception of rubbing the liver on the sensitive tissues of the mouth, all have proven invalid. As more reference material and standards became available, chemical and immunochemical methods have emerged.

Bioassays have one common disadvantage: the lack of specificity for individual toxins. Alternative methods based on immunochemistry (Hokama et al.,1977) are applicable to screening fish in the marketplace. The original assay, a radioimmunoassay (RIA) for ciguatoxin, was developed using antibodies produced against a conjugate of human serum albumin and ciguatoxin (isolated from toxic moray eel) injected into sheep and rabbit. This assay was used successfully to test ciguatera and to screen for toxic amberjacks (*Seriola dumerili*) where 15% of the fish were rejected during a 2-yr study on the Hawaiian market (Kimura et al.,1982). Despite this success, the assay was not suitable for routine use due to high cost, instrumentation requirements, and time involvement.

In 1983, a competitive enzyme immunoassay (EIA) commonly called the 'stiektest' was developed using the polyclonal antibody used in the RIA, and evaluated on Hawaiian reef fishes (Hokama et al.,1983; Hokama et al.,1984; Hokama,1985). As with its predecessor, this antibody demonstrated close structural similarity of CTX, MTX, brevetoxin, and OA. EIA used liquid-paper applied to bamboo sticks to isolate and bind the toxins (Hokama,1985). This assay was able to distinguish between toxic and nontoxic fish. Test results revealed a high number of false-positives, although no false-negatives were observed (Hokama et al.,1987; Hokama & Miyahara,1986).

The stick test was modified further using monoclonal antibodies specific for CTX, OA, and a synthetic fragment of OA, that are more specific than the sheep antibody (Hokama et al.,1990; Hokama et al., 1992; Hokama et al.,1986). This antibody gave peak titers of 1.5ng, 10ng and 50ng, respectively, for CTX, the fragment of OA, and OA (Hokama et al.,1992). Competitive inhibition analyses showed that 4ng purified CTX blocked completely the antibody reaction with crude CTX, OA and the fragment of OA at similar concentrations (approximately 50ng). This assay was used to test fish specimens

from documented cases of ciguatera (Hawaii Department of Health) with 98% agreement (Hokama et al.,1989). A preliminary collaborative evaluation study of the rapid enzyme immunoassay stick test was conducted (Ragelis, 1987,1988). Eight of the nine laboratories involved obtained results within acceptable limits for each of 3 fish cake samples homogenized with ciguatoxin. The relative standard deviation of reproducibility (RSD_R) was 23–30%. Due to the lack of a chemically identifiable standard, the full collaborative study was not conducted.

This assay was modified to a solid-phase immunobead assay format (Hokama,1990) commonly known as the 'paddle test', using bamboo paddles coated with liquid paper. This format was used to test 26 cases of ciguatera with 100% agreement. In a study comparing the stick and paddle tests, 436 specimens with varied levels of toxicity showed 80% agreement (Hokama,1990).

Patents covering the stick and paddle tests were purchased by HawaiiChemtect International. The original format was modified to an innovative rapid solid-phase immunobead assay (S-PIA, Ciguatect™) for ciguateric toxins (CTX) and diarrheic shellfish poisoning (DSP) outbreaks (Park & Goldsmith,1991; Park et al.,1992b). Toxins are determined by binding them to a membrane attached to a plastic strip and exposing the toxin-ladened membrane to a monoclonal antibody-colored latex bead complex which has a high specificity for the toxins of interest. The intensity of the color on the membrane denotes the toxins. CTX toxicity potential can be determined directly on edible tissue or following specific extraction procedures. The method has been used to evaluate CTX potential in fish obtained from Hawaii, Australia, and the Caribbean (Park et al.,1992b). The Ciguatect™ test kit has been compared to the mouse assay for the detection of toxic fish, i.e. fish of tropical origin for sale in Canada (Todd *et al.*,1992) and fish collected from St. Thomas, U.S. Virgin Islands (Dickey et al.,this memoir). Both studies reported a high percentage of fish toxic to the mouse and positive for CTX-related toxins by the Ciguatect™ test kit or following a rapid extraction and purification procedure. CTX-related toxins are present in a significant number of fish; however, the toxicological or public health significance is unknown, i.e., would the toxin(s) present (profile, potency and concentration) pose a significant risk for acute poisoning and/or chronic toxicity? Todd and co-workers used 135–250g equivalent fish flesh for injection into the mouse where 85% of the mice died within 24hours. Dickey et al. (this memoir) used 45–180g equivalent fish flesh and 67% of the mice died within 48 hours. Interpretation of mouse assay results must be made with caution, however. Mouse toxicity results have been useful in confirming toxins in ciguatera outbreaks, although the mouse is relatively resistant to CTX. Because of the lack of specificity, the mouse bioassay should not be used to predict ciguatoxicity. This was particularly apparent with the Dickey et al. study where for 22% of the specimens one animal died within a short time frame and the duplicate animal survived 48 hours. 43%, of the animals that died, died within 30min. Short death times (<30min) with the mouse are not considered ciguatoxic. Additionally, the statistical procedure used by the authors can only be applied when the method used for comparison by definition is 100% accurate for sensitivity and specificity (Riegelman & Hirsch,1989). The mouse assay is unlikely to be 100% accurate in determining ciguatera toxicity. As was pointed out by Hoffman et al. (1983) and Vernoux (1993), the mouse can have utility when symptomatology is used as well (symptomology was used as a criteria in the Dickey et al. study). A preferred procedure for the evaluation of a test method is outlined below.

For those products where additional testing is desired, possibly for samples testing positive, the University of Arizona and HawaiiChemtect International have developed a rapid extraction method (REM™) capable of extraction and partial purification of ciguateric toxins in <30 mins (Park et al.,1992a). The REM procedure isolates and purifies CTX-related toxins on the same chemical basis as used in more exhaustive CTX extraction procedures. For the REM™, toxins are extracted with a chloroform:water:methanol mixture and partitioned into selected phases by varying polarity. When the REM™ is used in combination with the Ciguatect™ test kit, the limit of detection for ciguatoxin, okadaic acid and related toxins is <0.05ng/g fish flesh. Also, at this point chemical methods based on thin layer (TLC) or high performance liquid chromatography (HPLC) technology can be used to confirm individual toxins.

Methods based on thin layer (TLC) and high performance liquid chromatography (HPLC) have been developed for selected individual toxins associated with CTX (Lee *et al.*, 1987; Legrand,1991; Dickey *et al.*, 1990). These methods can be applied as a regulatory tool where

TABLE 1. Precision parameters of collaborative data for solid-phase immunobead assay (Ciguatect™) determination of ciguatoxins and related polyether compounds in parrot fish, surgeon fish and amberjacks from the Hawaii Island.

	mean	S_r	S_R	RSD_r (%)	RSD_R (%)
	Fish Fillets				
Parrot Fish (*Scarus* sp.)	1.2	0.16	0.53	13.5	44.4
Surgeon Fish (*Ctenochaetus* sp.)	1.7	0.15	0.50	9.0	29.7
Amberjack (*Caranx* sp.)	3.6	0.15	0.51	4.3	14.3
	REM Extracts				
Parrot Fish (*Scarus* sp.)	3.1	0.18	0.37	5.8	11.9
Surgeon Fish (*Ctenochaetus* sp.)	3.8	0.18	0.38	4.8	9.9
Amberjack (*Caranx* sp.)	4.9	0.18	0.37	3.7	7.6

S_r= Standard deviation of repeatability
S_R = Standard deviation of reproducibility
RSD_r= Relative standard deviation of repeatability
RSD_R = Relative standard deviation of reproducibility

sophisticated laboratory facilities are available. HPLC techniques have been applied to analysis of okadaic acid in fish and shellfish (Gamboa *et al.*, 1992; Yasumoto,1985; Lee et al.,1989; Dickey et al.,1990). Park and co-workers (unpubl. data) have developed a TLC method for okadaic acid in fish tissue and dinoflagellate cultures. Specificity of this methodology is enhanced by exhaustive purification of toxins extracted from fish tissue. Unfortunately, CTX and okadaic acid develop similarly on TLC. These methods, although not suitable for routine screening programs, could play an important role in confirming the presence of individual toxins in fish products.

HPLC methodology have been reported for ciguatoxin and several analogues (Murata et al.,1990; Lewis et al.,1991; Lewis & Sellin,1992). These studies reported four major ciguatoxins. Legrand (1991) and co-workers (Legrand et al.,1990) used HPLC methodology to isolate multiple ciguatera toxins from wild *Gambierdiscus toxicus* and toxic herbivorous and carnivorous fish.

METHOD VALIDATION

Any method intended to be used in a seafood safety monitoring program must pass stringent in-house evaluation and be validated through an inter-laboratory study to determine precision (reproducibility, repeatability) and accuracy (recovery) parameters of the method. Method validation programs are administered by AOAC International (AOAC) and International Union for Pure and Applied Chemistry (IUPAC). These validation programs include two phases. The first phase is a ruggedness test or feasibility (mini-collaborative) study. Acceptable results in this phase lead to a collaborative study (Phase II). These studies (mini- and full-collaborative) involve the distribution of coded samples (in duplicate) of fish, preferably authentic ciguatera fish poisoning specimens, to participating laboratories. Known amounts of specific standards are also added to some of the samples. The samples are analyzed following exact method protocols and results returned to the study organizer. Phase I of the validation process, i.e., mini-collaborative study, was carried out for the S-PIA using fish fillets and REM™ extracts (Park et al.,1992a,b). The AOAC/IUPAC inter-laboratory mechanism was used. The precision of the S-PIA (Ciguatect™) to detect CTX has been evaluated through analysis of toxic and non-toxic fish fillets (amberjack, surgeon, and parrot fish) and REM™ extracts of the same fish obtained from fishing areas around the Hawaiian Islands. Toxicity potentials of purified extracts were determined using the mouse and brine shrimp (*Artemia* sp.) assays. The analysis showed acceptable repeatability and reproducibility parameters (Table 1). University of Arizona, FDA and NMFS laboratories participated in the study. The study confirmed excellent performance and interpretation of results, and demonstrated acceptable precision parameters (Park et al.,1992a,b). A full-collaborative study of the test kit is recommended. The validation study will include test portions of naturally and artificially contaminated fish with ciguatoxin and okadaic acid. Toxicity potentials will be determined using the mouse and brine shrimp assays.

SEAFOOD SAFETY MONITORING PROGRAMS

An effective food safety monitoring program comprises: 1, monitoring fish harvesting areas for CTX; 2, establishment of regulatory limits, and; 3, screening commercial fish products. Unacceptable product can be further tested to identify the toxin(s).

MONITORING FISH HARVESTING AREAS

Since seafoods commonly associated with

ciguatera poisoning outbreaks are associated with highly mobile fish, collecting and testing such fish alone could provide misleading information so testing less mobile species (e.g. invertebrates) should be included.

The Ciguatect™ S-PIA screened 36 species of nearshore invertebrates off the Island of Hawaii for ciguatoxin and related polyethers (Fig.1) (R.G. Kvitek, Moss Landing Research Laboratories, and D.L. Park, University of Arizona, unpubl. data). Specimens included snails, sea urchins, sea cucumbers, crabs, brittle stars, bivalves, and zoanthids. Invertebrates were collected at 6 'toxic' locations along the Kona coast, and at 3 'non-toxic' sites along the Hamakua coast where there had been only one reported case of ciguatera since 1980.

A significant positive correlation between assay results and site-specific ciguatera history was found for the cowry *Cypraea maculifera* (Fig.2). While assay results for most other species indicated low or no ciguatoxin, cone snails (*Conus*), ophiuroids (*Ophiocoma*) and sea cucumbers (*Holothuria*) tested positive frequently. There was no correlation, however, for these three genera between assay results and site history. These results suggest that invertebrates, particularly grazers and deposit feeders, and especially cowries, accumulate ciguatoxins and related polyether compounds at sites known for ciguatera fish poisoning outbreaks and have the potential utility of being bio-indicators of reef toxicity. This marine specimen, or other invertebrates native to other areas, could be an integral part of the ciguatera monitoring program.

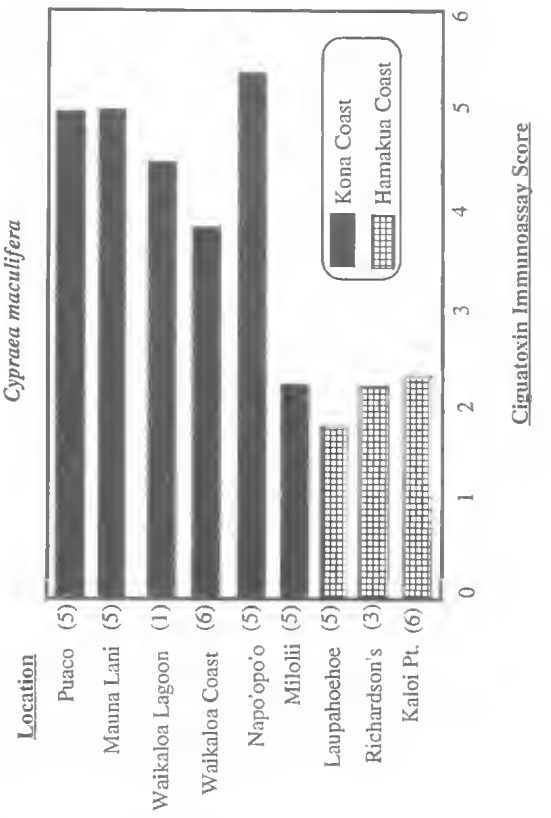

FIG. 2. Ciguatoxin immunoassay scores for *Cypraea maculifera* from Kona and Hamakua Coasts of Hawaii. Color intensity on the test strip assigned a value between 0-6 where 0 = nondetectable and 5 = color intensity equal to 5ng okadaic acid. Numbers in () indicate number of individual specimens tested and values pooled.

Establishment of Regulatory Limits

Based on mouse and mosquito bioassay data, several levels of concern have been proposed. Since multiple toxins of varied toxin potential are involved with CTX poisoning, it is not practical to use a single compound for this regulatory limit. Historically, a seafood safety monitoring program for ciguatera has been hampered by the lack of reference standards, particularly ciguatoxin. Okadaic acid is the only toxin associated with CTX poisoning in sufficient quantities to serve as a reference standard. Toxicity of okadaic acid, however, is significantly lower than ciguatoxin where acute mouse toxicity potentials for ciguatoxins and okadaic acid are 0.45 and 210μg/kg, respectively. The term okadaic acid equivalents (OAE) could be used, however, to *standardize analytical methods* and in the *establishment of regulatory limits*, provided the relative potencies of all toxins involved with the CTX phenomenon are used in calculation of action levels. For this to be feasible, the test employed must recognize all toxins involved in the poisoning in a similar manner and the action level focus on the toxin of highest potency. Again, the term OAE would be used because multiple toxins are involved in the poisoning.

Screen Fish in the Marketplace/Commercial Channels

Any method for screening marketplace seafoods must: a, be easy to use and interpret; b, be rapid, i.e., able to test a large number of samples in a short time; c, accurately differentiate between toxic and non-toxic samples; d, have low cost; e, be available in quantities to meet private, industrial, and regulatory agency testing

demands; and f, where feasible, confirm toxin identity.

The S-PIA method (Ciguatect™) has high potential for screening market place fish. When fully validated, the kit may be used at the harvesting, processing, distribution, retail or other point through the marketing route. Testing fish early after capture is recommended, since this will minimize cost expended for the product and potential economic loss to the industry. The kit can be used on-board fishing vessels, at receiving docks, processing plants, distributing organizations, retail outlets, consumers, and regulatory agencies. The self-contained assay is available as a single analysis kit designed for non-laboratory use by untrained personnel. Organizations conducting large numbers of analyses would be more inclined to use the laboratory kit which contains sufficient material for >50 tests.

The Seafood Safety Monitoring Program would involve large-scale testing of fish according to an acceptable sampling plan. Fish or lots testing negative to the screening procedure would be allowed to proceed normally in commercial channels. Each point identified above would be a quality control point. Product testing positive for toxic potential would be diverted to lower risk uses or retested to confirm toxic potential. This can be done by using the REM procedure which isolates, purifies and concentrates the toxins before retesting or by using alternative test methods for specific toxins.

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